Tailoring chitosan/collagen scaffolds for tissue engineering: effect of composition and different crosslinking agents on scaffold properties.

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ABSTRACT

Chitosan/collagen (Chit/Col) blends have demonstrated great potential for use in tissue engineering (TE) applications. However, there exists a lack of detailed study on the influence of important design parameters (i.e, component ratio or crosslinking methods) on the essential properties of the scaffolds (morphology, mechanical stiffness, swelling, degradation and cytotoxicity). This work entailed a systematic study of these essential properties of three Chit/Col compositions, covering a wide range of component ratios and using different crosslinking methods. Our results showed the possibility of tailoring these properties by changing component ratios, since different interactions occurred between Chit/Col: samples with Chit-enriched compositions showed a hydrogen-bonding type complex (HC), whereas a self-crosslinking phenomenon was induced in Col-enriched scaffolds. Additionally, material and biological properties of the resultant matrices were further adjusted and tuned by changing crosslinking conditions. In such way, we obtained a wide range of scaffolds whose properties were tailored to meet specific needs of TE applications.

Keywords: collagen, chitosan, scaffolds, crosslinking (XL), tissue engineering (TE).

1. INTRODUCTION

Regenerative medicine is an emerging field that aims to improve or repair the performance of a damaged tissue or an organ. Cells are used in combination with 3D scaffolds, based on biocompatible biomaterials, for developing suitable tissue engineering (TE) constructs (Langer, 2000; Langer & Vacanti, 1993). The main function of TE scaffolds, i.e. cell supports, is to mimic the function of extracellular matrix (ECM) which not only provides an appropriate mechanical environment for cells, but also supplies signals that direct cell attachment, proliferation, differentiation and metabolism. The choice of biomaterials and the selection of experimental conditions for design of these scaffolds are essential parameters in assuring the appropriate setting for cells to grow and proliferate within these 3D matrices (O'Brien, 2011; Ou & Hosseinkhani, 2014).

Proteins and polysaccharides are considered promising natural molecules for the design of 3D scaffolds with the required characteristics for TE applications (Chen et al., 2012; Fischbach, Kong, Hsiong, Evangelista, Yuen & Mooney, 2009; Gurski, Jha, Zhang, Jia & Farach-Carson, 2009). This work has been focused on the use of collagen (the most abundant protein in the extracellular matrix, ECM) and chitosan (a natural polysaccharide structurally similar to glycosaminoglycans) as main components for the development of 3D porous scaffolds for potential applications in a variety of tissue engineering procedures.

It is well documented that both collagen (Col) and chitosan (Chit) have great potential in the design of a variety of bioactive materials for different biomedical purposes (Croisier & Jérôme, 2013; Szot, Buchanan, Freeman & Rylander, 2011). Collagen possesses excellent biocompatibility and biodegradability and comprises the repetitive array of receptor-recognition motifs essential for cell interaction via specific

75 collagen-binding β_1 integrins, such as GFOGER motif (G: glycine; F: phenylalanine; O:
76 hydroxylproline; E: glutamate and R: arginine) (Knight, Morton, Peachey, Tuckwell,
77 Farndale & Barnes, 2000; Yannas, Tzeranis, Harley & So, 2010). Consequently,
78 collagen-based scaffolds should not only mechanically support the native tissue during
79 repair, but also play an important role in providing essential signals to influence cell
80 activity (Yannas, Tzeranis, Harley & So, 2010).

81 Chitosan, a biocompatible and biodegradable polymer with structural
82 characteristics similar to the glycosaminoglycans, exhibits various interesting biological
83 properties, such as biocompatibility, good interaction with cell membranes and lack of
84 immunogenicity (Croisier & Jérôme, 2013; Kievit et al., 2010). It has been reported that
85 this natural polysaccharide can stimulate the activity of growth factors, contribute to the
86 maintenance of cell phenotypes, especially in terms of its morphology, and play an
87 important role as a structural component of scaffolds for soft and hard tissue
88 regeneration (Croisier & Jérôme, 2013). The chitosan-based 3D matrices have showed
89 both *in vitro* cytocompatibility and *in vivo* biocompatibility (Guzman et al., 2014). One
90 of the interesting features of chitosan is its cationic nature in acidic solutions due to its
91 protonated primary amine groups. The cationic charges allow chitosan to form water-
92 insoluble ionic complexes with a variety of polyanionic substances (Croisier & Jérôme,
93 2013), for example, with negatively charged collagen. It should be mentioned that in the
94 natural ECM, proteoglycans and glycosaminoglycans have important roles in
95 intertwining with the fibrous structure of collagen to obtain mechanical stability and
96 compressive strength (Fernandes, Resende, Tavares, Soares, CastroI & GranjeiroI,
97 2011). Therefore, it seems interesting to develop mixed collagen-chitosan scaffolds in
98 order to create more suitable biomimetic microenvironments for cells compared to those
99 provided by pure Col or Chit matrices.

100 It is well known that various chemical crosslinkers are frequently introduced in
101 the production process (Davidenko, Campbell, Thian, Watson & Cameron, 2010;
102 Denkbas & Ottenbrite, 2006) to enhance the structural stability of natural polymer-
103 based scaffolds. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride
104 (EDAC) in the presence of N-hydroxysuccinimide (NHS), and sodium tripolyphosphate
105 (TPP) are two of the most commonly used chemical agents which work in a different
106 manner and on different types of molecules. EDAC activates carboxylic acid groups on
107 the collagen-type molecules forming ‘‘zero length” crosslinks with free primary amine
108 groups of polypeptide chains. TPP is widely used to improve the stability of chitosan-
109 based materials and acts through ionic interactions with chitosan amine groups. Both
110 cross-linkers are considered to be non-toxic and biocompatible, features which favour
111 their use in the enhancement of scaffold stability. Therefore, essential material and
112 biological properties of the resultant matrices obtained with different component
113 fraction might be further adjusted and tuned by use of these chemical treatments (alone
114 or in combination).
115 Due to evident potential of collagen-chitosan blends, a variety of these kinds of
116 composite biomaterials, including porous matrices, have been developed for different
117 biomedical purposes (Ti et al., 2015; Zeng et al., 2014). Cytocompatibility and some
118 physical properties of blended scaffolds were determined and compared to those of pure
119 Chit or Col samples (Fernandes, Resende, Tavares, Soares, CastroI & GranjeiroI, 2011).
120 There is, however, a lack of detailed and systematic study on the influence of
121 important design parameters, such as component ratio (over a wide range of values) and
122 the use of the different chemical treatments (crosslinking methods), on the essential
123 structural, physico-chemical, mechanical and biological properties of the scaffolds.
124

Therefore, the main objective of this study was to determine the effect of changing composition and crosslinking methods on both the essential material properties and the biological suitability of scaffolds obtained from different collagen-chitosan mixtures. Three Chit/Col compositions, namely Chit/Col=80/20, 50/50 and 20/80 (w/w%) were selected to cover a wide range of component ratios. This choice was based on the fact that depending upon the component fraction in the mixture and experimental conditions, different kind of interaction could take place between these two polymers (Taravel & Domard, 1996). It was expected, therefore, that both polymers would not only contribute to the final composite properties with their own characteristics, according to their content in the blend, but also would interact with each other, and form different kinds of complexes which would subsequently influence the mechanics and dissolution kinetics of the resultant materials.

Our expectations were based on reports (Taravel & Domard, 1993, 1995, 1996) showing that principally two kinds of interactions can arise between these two polymers when they are in contact with water. The first is electrostatic in nature and corresponds to the formation of a polyanion/polycation (PA/PC) complex between the two types of polyelectrolytes. Theoretically, the best conditions for this interaction should be achieved under the stoichiometric ratio of 18.5% (w/w) between chitosan and collagen. In this work 20Chit/80Col mixture was selected to reproduce this condition. (Taravel & Domard, 1993, 1995, 1996). In addition to this, a hydrogen-bonding type complex (HC) may also be formed, especially in the presence of a large excess of chitosan. 80Chit/20Col composition was chosen to recreate this condition and 50Chit/50Col mixture was set to represent the medium state between two extreme cases. It was reported that in the PA/PC complex the triple helix structure is preserved and even

149 reinforced by the presence of chitosan, but in the HC complex chitosan chains seem to
150 induce the destabilisation of the triple helix organization and so denature the collagen.
151 Taking into account the possibility of these types of interactions occurring
152 between collagen and chitosan, and by changing component ratios, scaffolds with
153 different swelling/degradation kinetics and different mechanical properties should be
154 expected to be formed. By changing crosslinking conditions using different chemical
155 treatments it was foreseen that a further variation of properties could be achieved.
156 Consequently, a wide battery of structures with different physicochemical behaviours
157 were obtained and characterized in terms of morphology, degradation, swelling,
158 mechanical properties, and also in terms of preliminary cytotoxicity to consider the
159 possibility of performing further biological studies.
160

161 **2. MATERIALS AND METHODS**

162 *2.1 Materials*

163 Type I microfibrillar collagen derived from bovine Achilles tendon, chitosan
164 (medium molecular weight, viscosity 200-800 cps, 75% deacetylated), *N*-
165 Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbo-diimide
166 hydrochloride (EDAC), sodium tripolyphosphate (TPP), lysozyme from chicken egg
167 white (40,000 U/mg), collagenase from *Clostridium histolyticum* type IA (125 U/mg),
168 methylthiazoletetrazolium (MTT) and dimethylsulphoxide (DMSO) were purchased
169 from Sigma-Aldrich (Barcelona, Spain). Acetic acid, absolute ethanol, potassium di-
170 hydrogenphosphate, dehydrated di-sodium hydrogenphosphate and hydrochloric acid
171 (HCl) were purchased from Panreac (Barcelona, Spain). Fetal bovine serum (FBS),
172 Dulbecco´s modified Eagle´s medium (DMEM), penicillin (50 U/mL), streptomycin (50

μg/mL), L-glutamine (200mM) gentamicin (50 μg/mL) and 0.05% trypsin/0.53 mM EDTA were purchased from Lonza (Barcelona, Spain).

2.2 Chitosan/Collagen scaffold preparation

Porous scaffolds based on chitosan/collagen mixtures were prepared using a freeze-drying process. Firstly, a pure solution of chitosan and a suspension of collagen were prepared at 1% (w/v) concentration in acetic acid. Chitosan was dissolved in 0.5M acetic acid and centrifuged (Ortoalresa, Digicen 20-R) at 3000 rpm for 5 min to remove any solid impurities. Collagen was allowed to swell in 0.05 M acetic acid at 4-8°C overnight to produce a 1% (w/v) protein suspension. The resulting suspension was homogenised on ice for 30 minutes at 20,000 rpm using an overhead homogenizer (Ultra-turrax® IKA T18 basic). Air bubbles were removed by centrifuging at 2500 rpm for 5 min (Hermle Z300, Labortechnik, Germany).

Three mixed scaffold compositions with different volume percentages (v/v) of polymers, namely, 80% chitosan and 20% collagen (80Chit/20Col), 50% chitosan and 50% collagen (50Chit/50Col), and 20% chitosan and 80% collagen (20Chit/80Col), were produced by blending the corresponding polymer mixture at 20,000 rpm for 10 min in an ice bath and then centrifuging at 2500 rpm for 5 min to remove air bubbles. Chitosan/collagen suspensions were then poured into 24 well cell culture plates, frozen at -20°C overnight and freeze-dried for 72 hours at -110°C to ensure their complete drying (Heto PowerDryLL1500 Freeze Dryer, Thermo Electron Corporation).

2.3 Scaffold crosslinking

Freeze-dried chitosan/collagen scaffolds were crosslinked (XL) to increase their strength and degradation resistance using different methods. In the first method

198 scaffolds were treated with a water-soluble carbodiimide (Park, Park, Kim, Song & Suh,
199 2002) by immersing them in 95% ethanol solution containing 33 mM EDAC and 6 mM
200 NHS for 2 h at 25°C (0.32 g EDAC/g scaffold and 0.35 g NHS/g scaffold,
201 approximately). After the crosslinking process, the samples were washed sequentially in
202 96% (v/v), 70% and 50% ethanol for 10 min in each solution, following by a final
203 washing with distilled water (10min x 3). In the second method the scaffolds were first
204 stabilized with ethanol by immersing in 96% and then 70% ethanol for 1.5 h in each
205 solution followed by washing with distilled water. Following this, the samples were
206 treated with 1% TPP solution in distilled water (w/v, pH 3.3) for 2 h at 25°C (0.5 g
207 TPP/g scaffold approximately) and thoroughly washed in distilled water (10min x 6).
208 Finally, a third crosslinking method was carried out combining EDAC and TPP.
209 Scaffolds were immersed in 95% ethanol solution containing 33 mM EDAC and 6 mM
210 NHS for 2 h at 25°C. After alcoholic and aqueous washings, the scaffolds were
211 immersed in 1% TPP solution for 2 h and finally washed with water. At the end of each
212 crosslinking method, the scaffolds were refrozen and re-lyophilized using the previous
213 freeze-drying cycle.
214
215 *2.4 Scaffold morphology*
216 Scaffold pore structure and pore size distribution were analysed by scanning
217 electron microscopy (SEM). Each sample was cut through the thickness of the scaffold
218 sheet and cross-section samples were then mounted on stubs and sputtered with an ultra-
219 thin layer of platinum for 2 min at 20 mA. The samples were then studied with a JEOL
220 5800 scanning electron microscope operating at 10 kV. The average pore size was
221 obtained by measuring the maximum and minimum diameter in the image section of at
222 least 30 pores randomly chosen in each sample.

223 Porosity values were obtained using the following equation (Kumar, Batra,
224 Kadam & Mulik, 2013):

225 $$P\ (\%) = (1-\rho_r) \times 100 \quad (1)$$

226 P being the porosity percentage value, and ρ_r the relative density value.

227 The relative density of each scaffold, ρ_r, was defined as:

228 $$\rho_r = \rho^* / \rho_s \quad (2)$$

229 where ρ^* represents the dry density of each freeze-dried sample, and ρ_s is obtained from
230 the known dry densities of solid collagen ($\rho_s = 1.3\ g \cdot cm^{-3}$) (Khor, 1997) and chitosan (ρ_s
231 $= 0.5\ g \cdot cm^{-3}$) (Chatterjee, Lee, Lee & Woo, 2009).

232 In order to calculate the ρ^* value, the mass and volume of each sample were
233 determined after the freeze-drying process, and changes in these parameters, caused by
234 the crosslinking process, were also evaluated using the following equations:

235 $$\text{Volume Shrinkage } (\%) = 100 \times [(V_0 - V)/V_0] \quad (3)$$

236 where V_0 and V are the volumes of the scaffold before and after crosslinking,
237 respectively;

238 $$\text{Mass Loss } (\%) = 100 \times [(m_0 - m)/m_0] \quad (4)$$

239 where m_0 and m are the masses before and after crosslinking, respectively.

240

241 *2.5 Dissolution study*

242 Crosslinked and non-chemically crosslinked (Non-XL) samples were hydrated
243 in PBS (Phosphate Buffer Saline, pH 7.4) at 37°C to evaluate their degree of
244 dissolution. Scaffolds were cut to approximate cuboids (8 × 8 × 18 mm), weighed prior
245 to the dissolution study ($W_{d'}$), and then immersed in 2 ml PBS at 37°C for up to 14 days.
246 At different time points they were removed, washed in a large volume of deionised
247 water to remove buffer salts, and dried at 37°C until constant mass was reached. Finally,

the samples were weighed (W_a) and the percentage weight loss was calculated as follows:

$$\text{Weight loss (\%)} = 100 \times [(W_{d'} - W_a)/ W_{d'}] \quad (5)$$

The pH value of the PBS was measured at each time point using a pH-meter (METROHM 654). Each sample was measured in quadruplicate.

2.6 Swelling study

This study was carried out in PBS. Scaffolds were cut to approximate cuboids (8 × 8 × 18 mm), weighed and then immersed in 2 ml PBS at 37°C for up to 14 days. At different time points, they were removed and two different measurements of their capacity to retain biological fluid were made. The first measurement was aimed at assessing the ability of the scaffold structure as a whole (the material itself together with the pore system) to absorb PBS. For this, at each time point, the samples were removed from PBS, shaken gently and then weighed without dripping (Wws). The second measurement was carried out after pressing and ‘‘drying” the same soaked samples between sheets of filter paper to remove the water retained in its porous structure (Wwm). In this way the swelling ability of scaffold material itself was determined. The scaffolds were then dried at 37°C until constant mass was reached (Wd).

The percentage of fluid uptake, in both cases, was calculated as shown:

$$\text{Fluid uptake of scaffolds \%} = [(Ww - Wd)/Wd] \times 100 \quad (6)$$

where Ww represents Wws or Wwm.

Each sample was measured in quadruplicate.

2.7 *Mechanical test*

A compressive stress-strain study of the scaffolds was carried out with a Hounsfield tester, using a 5 N load cell for the study and applying a constant compressive rate of 5 mm min^{-1}. Prior to the compressive test, scaffold cuboids (8 × 8 × 18 mm) were immersed in distilled water at room temperature for 1h. Compression platens were lowered to make contact with the samples, so producing a detectable and small load, and then the samples were compressed until densification occurred, at which point the tests were stopped. Stress-strain curves were drawn and used to calculate the linear elastic (Young´s) modulus via linear regression of the initial linear region of the respective curve (strain ranged approximately between 0.05-0.2). Each scaffold composition was assayed in quintuplicate, and three consecutive tests were also performed on each sample to study its capacity to maintain the mechanical properties after several compressions.

2.8 *In vitro collagenase and lysozyme degradation tests*

Two digestion tests were performed in the presence of collagenase and lysozyme, respectively, in order to study the biological stability of the scaffolds. Scaffolds were cut to approximate cuboids (8 × 8 × 18 mm) and were weighed before the degradation studies (W_b). For the collagenase test, the samples were immersed in 2ml PBS containing 160 μg/ml of collagenase while, in the case of lysozyme, the samples were immersed in 2ml PBS containing 1 mg/ml of lysozyme. The samples were then incubated at 37°C for up to 10 days. At different time points, they were removed, washed in a large volume of deionised water and dried at 37°C until constant mass was reached. Finally, the samples were weighed (W_e) and the percentage weight loss was calculated as follows:

$$\text{Weight loss (\%)} = 100 \text{ x } [(W_b - W_e)/ W_b] \quad (7)$$

297 Each study was performed in quadruplicate.

298

299 2.9 *Cell culture assays*

300 *2.9.1 Cell line and maintenance*

301 Human breast adenocarcinoma MCF-7 cells were selected for preliminary
302 testing of the cytocompatibility of the prepared structures. MCF-7 cells were obtained
303 from Dr. von Kobbe between 20 and 25 passage numbers, whose original source was
304 ATCC®. Cells were maintained in Dulbecco's modified Eagle medium, supplemented
305 with 10% heat inactivated FBS, penicillin (50 U/mL), streptomycin (50 μg/mL), L-
306 glutamine (200 mM) and gentamicin (50 μg/mL) in a humidified incubator at 37°C and
307 5% CO_2 atmosphere (HERA cell, Sorvall Heraeus, Kendro Laboratory Products Gmbh,
308 Hanau, Germany). The cells were plated in a 75-cm^2 flask (Sarstedt Ag and Co.,
309 Barcelona, Spain) and were passaged when reaching 95% confluence, by gentle
310 trypsinization. According to the results obtained from characterization studies, the
311 scaffolds which offered better bulk characteristics were tested with cells. Thus, TPP-XL
312 and EDAC+TPP-XL samples were selected for 80Chit/20Col and 50Chit/50Col
313 compositions, whereas Non-XL and EDAC+TPP-XL samples were chosen for
314 20Chit/80Col mixture.

315 *2.9.2 Cell culture and seeding on chitosan/collagen scaffolds*

316 Prior to the seeding, the scaffolds were cut to approximate cuboids (8 × 8 × 9
317 mm), sterilized with ethanol and pre-incubated with DMEM supplemented with
318 25%FBS for 30min at 37°C. Samples were then placed on to Petri plates covered with
319 sterilized parafilm for them to be inoculated with cells. $2x10^5$ MCF-7 cells in 200 μl
320 medium with 10% FBS were seeded on to the scaffold surface and incubated for 1h to
321 achieve cell attachment to the scaffolds. After this time, the inoculated samples were

322 moved to Petri plates containing 10 ml of DMEM with 10% FBS, and were incubated
323 for up to 5 days. Some samples were not inoculated with cells but followed the same
324 protocol for use as controls.

325 *2.9.3 Cell proliferation and growth kinetics study*

326 Proliferation of MCF-7 cells on the scaffolds was quantitatively assessed with 3-
327 [4,5-dimethylthiazol-2-yl]-diphenyltetrazolium bromide (MTT) (Seda Tigli, Karakecili
328 & Gumusderelioglu, 2007) at different culture periods of up to 5 days. At different
329 culture times, control and inoculated samples were removed from the Petri plates and
330 placed on to 24-well plates containing 1ml medium per well. Then, 100 µl MTT
331 solution (5 mg/ml in PBS) was added to each well. The samples were incubated at 37°C
332 for 2h. After this the medium was removed and 200 µl DMSO were added to dissolve
333 formazan crystals. The absorbance was measured by a spectrophotometer at 570 nm
334 (Varioskan, Thermo Fisher Scientific, Barcelona, Spain). MTT assay was also applied
335 to the scaffolds without cells and the signal obtained was subtracted from the inoculated
336 scaffold signal. Exponential cell growth was assumed and a specific growth rate (µ) was
337 determined by fitting the following equation to the absorbance data (Seda Tigli,
338 Karakecili & Gumusderelioglu, 2007):

339 $$\ln (A/A_0) = \mu (t-t_0) \quad (8)$$

340 where A_0 is the initial absorbance at t_0 (h), A is the absorbance at t (h) and µ is the
341 specific growth rate (h^{-1}). Four samples of each composition (n = 4) were tested at each
342 experiment, and the assay was performed in triplicate.

343

344 2.10 *Statistical analysis*

345 Statistical comparisons were performed using one way analysis of variance
346 (ANOVA) followed by Bonferroni post hoc analysis with computer software SPSS

19.0. Values of $p<0.05$ and $p<0.01$ were considered significant and very significant, respectively.

3. RESULTS

3.1 Scaffold morphology

The influence of composition and crosslinking methods on pore morphology was studied by SEM. Fig.1 shows SEM low magnification images of transverse scaffold sections.

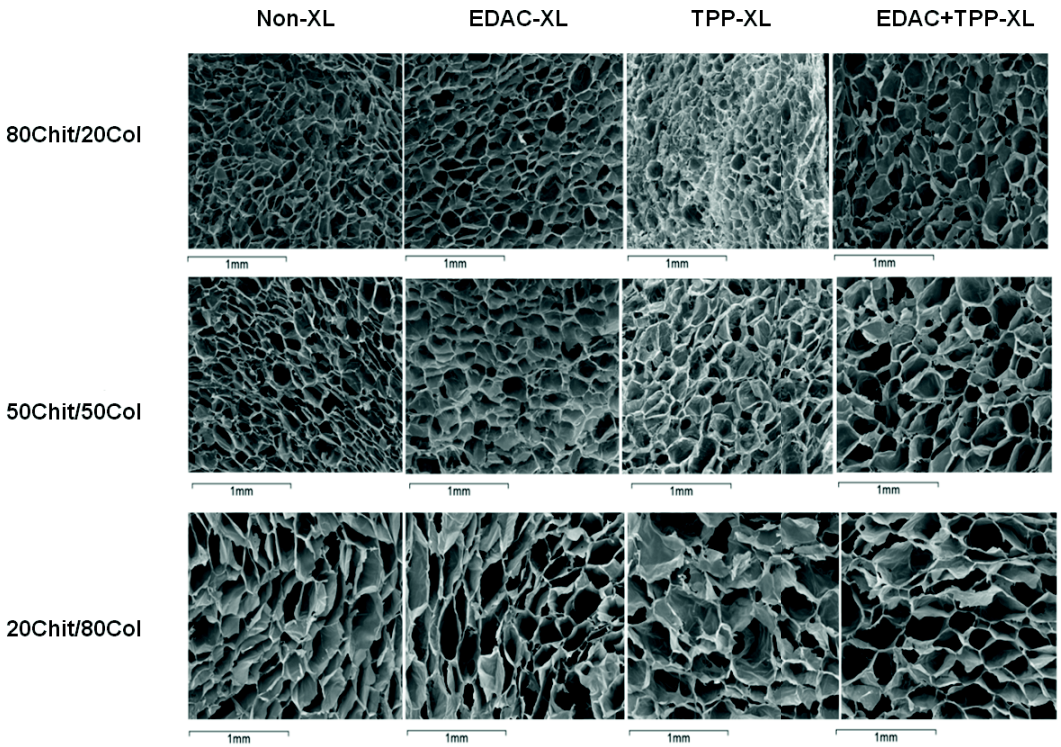


Fig. 1. SEM micrographs of the transversal sections of freeze-dried scaffolds with different compositions and different crosslinking treatments.

A continuous structure of interconnected pores with mainly uniform distribution was obtained in the three compositions, and was retained after crosslinking treatments. It was observed that pore shape tended to be rounded in all cases, since the same

freezing conditions were used for their production. The average pore size and porosity values are shown in Table 1. The pore dimensions estimated from SEM microphotographs were mostly in the range of 120-300 μm for all studied compositions and XL status. All scaffolds were highly porous; porosity values in the interval of ~ 96-99% were obtained for all compositions. It was observed that the variation in composition affected scaffold dimensional properties: for Non-XL samples the pore size and porosity increased with the increase of collagen percentage in the scaffold mixture.

Table 1. Morphological characteristics of the scaffolds pre- and post-crosslinking.

Composition	Porosity (%)	Pore size (μm)	Shrinkage (%)	Mass loss (%)
80Chit/20Col nX	97.9 ± 0.2	168 ± 54		
80Chit/20Col EDAC	97.9 ± 0.3	197 ± 57	(-)3	(-)3
80Chit/20Col TPP	95.9 ± 0.4*	120 ± 42*	43*	(-)10*
80Chit/20Col EDAC+TPP	96.2 ± 0.4*	191 ± 45	38*	(-)12*
50Chit/50Col nX	98.4 ± 0.1	143 ± 44		
50Chit/50Col EDAC	98.02 ± 0.08*	191 ± 47	18*	(-)4
50Chit/50Col TPP	97.8 ± 0.2*	194 ± 52	22*	(-)10
50Chit/50Col EDAC+TPP	97.8 ± 0.1*	258 ± 60	22*	(-)9
20Chit/80Col nX	98.8 ± 0.1	262 ± 79		
20Chit/80Col EDAC	99.06 ± 0.05*	248 ± 73	(-)3	21*
20Chit/80Col TPP	98.7 ± 0.1	305 ± 80	20*	16*
20Chit/80Col EDAC+TPP	98.77 ± 0.08	281 ± 72	4	3

* Indicates a statistical difference ($p<0.05$) in comparison to a non-chemically crosslinked sample of the same composition. Values of porosity and pore size are presented as mean ± standard deviation

Regarding the possible influence of crosslinking method over pore size and porosity, it was observed that EDAC and EDAC+TPP treatments did not introduce any

substantial changes in pore size compared to Non-Xl samples. However, TPP treatment caused a significant ($p<0.05$) reduction in the pore size and in porosity values of the scaffolds with the highest chitosan content (80Chit/20Col) (Fig.1 and Table 1).

3.2 Dissolution study

The resistance of scaffolds to dissolution in PBS at 37ºC was strongly dependent on the composition and crosslinking treatment, as shown in Fig 2.

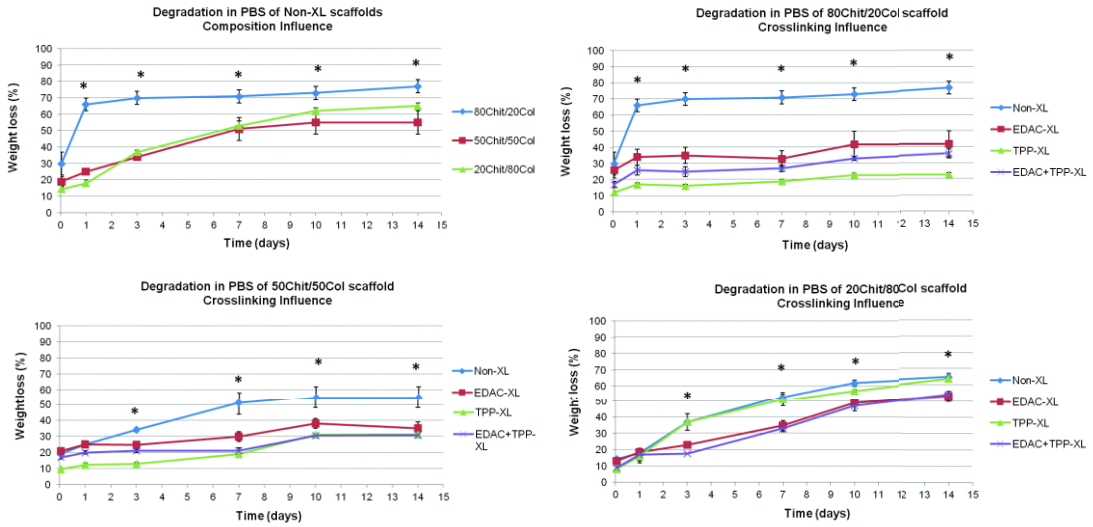


Fig. 2. Weight loss (%) of scaffolds in PBS at 37ºC according to composition and crosslinking status. * means that statistical differences were found ($p < 0.05$) ($n = 4$; bar charts represent standard deviation values).

For Non-XL samples (Fig 2) the percentage mass loss was significantly higher for scaffolds with the highest chitosan content (80Chit/20Col). The differences in the dissolution profiles were more noticeable at the early incubation stage (up to one week) but on day 14 all Non-XL matrices reached a similar level of dissolution, 50Chit/50Col and 20Chit/80Col compositions being more stable with 55 and 63% of mass loss, respectively, compared to ~80% dissolution for 80Chit/20Col.

Carbodiimide and TPP treatments generally increased the degradation resistance of all scaffold compositions in a biological fluid (Fig.2) but the level of stabilisation was highly dependent on scaffold composition and on XL method. For scaffold with the highest Chit content (80Chit/20Col) TPP treatment was the most effective, followed by combination of EDAC+TPP and EDAC alone (23% , 36% and 40% mass loss after 14 days, respectively). For the 50Chit/50Col composition, all crosslinking methods provided similar values (~33%) of scaffold degradation after 14 days in PBS, TPP being the most effective at early incubation phase (up to 7 days). In the case of collagen enriched composition (20Chit/80Col), TPP treatment showed no effect on scaffolds resistance to dissolution as no significant differences were observed between non-XL and TPP-XL samples. However, the carbodiimide chemistry, alone or in combination with TPP, enhanced scaffold resistance to dissolution, thus providing the lowest degradation values after these treatments.

The hydrolytic degradation of Non-XL crosslinked and crosslinked scaffolds did not cause a significant change in pH value, this being maintained in a range of 6.5 and 7.8 throughout the whole experiment.

3.3 Swelling study

The results of scaffold PBS-absorption ability are presented in Fig. 3, where swelling characteristics related to fluid retained both by the whole scaffold structure (overall uptake), and by the scaffold material itself, are displayed. In general, all scaffolds showed good capacity for overall PBS uptake. However, differences were observed according to the composition and crosslinking method employed.

In the case of Non-XL samples, the higher the chitosan content the bigger was the value of the overall fluid uptake at the early incubation time: 80Chit/20Col scaffolds

reached the maximum swelling degree (~11000%) after the first 24 hours, which was twice higher than for 50Chit/50Col matrices, and almost four times bigger than in the case of 20Chit/80Col (Fig. 3). However, after this time point both chitosan enriched compositions progressively lost their PBS absorption properties, these reaching the minimum values at day 14: in the case of 80Chit/20Col the decrease amounted to ~6 fold whereas for 50Chit/50Col to~10 times. This reflects pore collapse and loss of scaffold material to the medium. An opposite tendency was observed in the case of scaffolds with the highest Col content: 20Chit/80Col Non-XL composition steadily increased its swelling capacity over a period of 14 days incubation and reached the maximum value (~6000%) at the end of the testing period (day 14).

In order to assess the influence of the crosslinking method on PBS uptake properties, samples treated with different reagents were compared at each composition. It was observed that all XL methods influenced scaffold swelling behaviour and that the crosslinking effect on PBS uptake was noticeably dependent on both the composition and the XL treatment. For example, swelling profiles of 80% and 50% chitosan-based scaffolds treated with EDAC showed that both compositions arrived at their maximum swelling degree (as a whole structure) within the first 24 hours and 3 days in PBS, respectively, followed by a gradual decrease of this parameter, which was more marked for 80Chit/20Col composition (almost twofold, Fig 3). TPP treatment, alone or in combination with EDAC, enhanced the overall swelling capacity of both Chit-enriched compositions, significantly increasing their values of PBS uptake. For 20Chit/80Col samples, very pronounced time increasing profiles of PBS absorption were observed after all the three crosslinking treatments, the combination of EDAC and TPP being the method providing the highest value of fluid uptake (~10000%). This value was almost twice higher than for any other composition treated by any XL method.

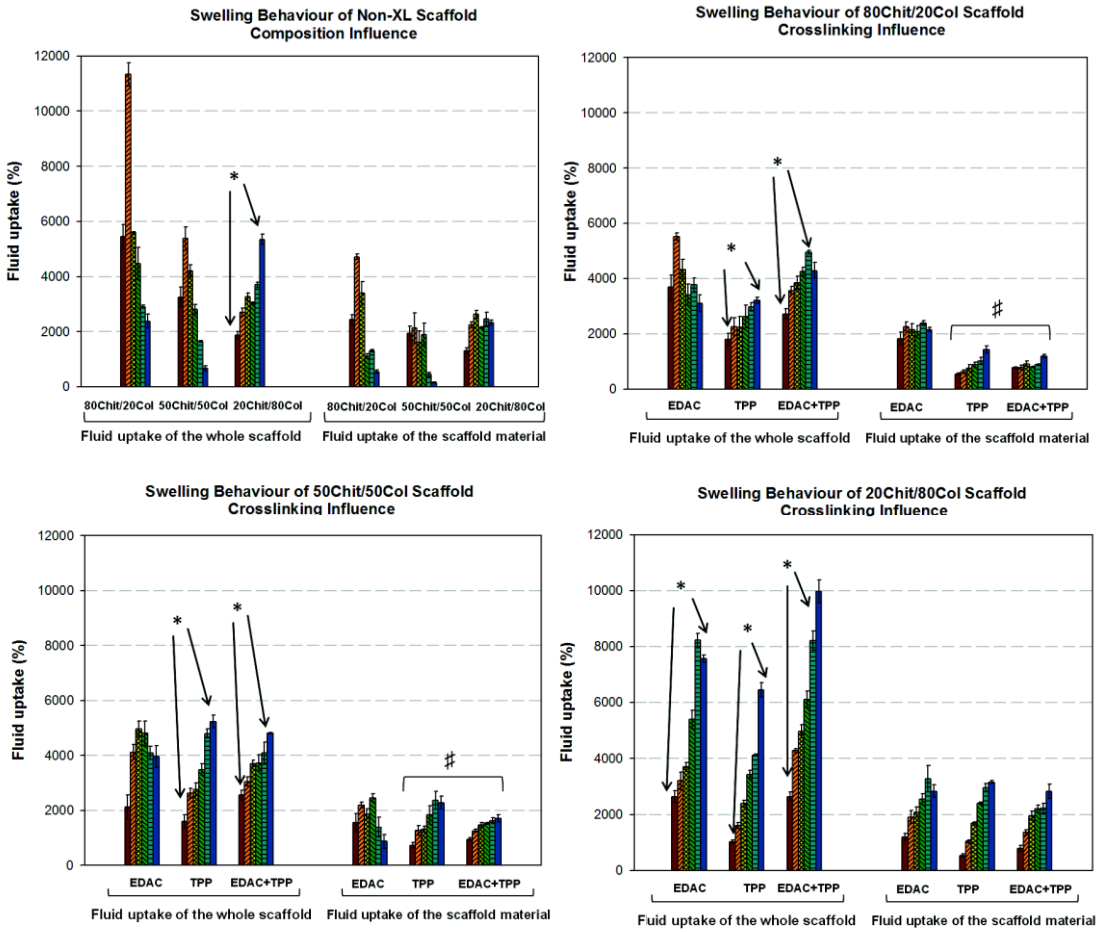


Fig. 3. Fluid uptake (%) of the whole scaffold and scaffold material in PBS at 37ºC according to composition and crosslinking status: after 1 hour ( ), after 1 day ( ), after 3 days ( ), after 7 days ( ), after 10 days ( ) and after 14 days ( ).* means that statistical differences were found ($p < 0.05$) between values at the beginning and the end of the experiment. ♯ indicates the compositions with significant restricted swelling behaviour due crosslinking action. (n = 4; bar charts represent standard deviation values).

Scaffold swelling abilities of material alone of untreated samples showed the same tendencies as those observed for the overall fluid uptake: for matrices with 50 and 80% of chitosan maximum fluid absorption occurred within the first 24 hours and then the percentage of material swelling fell gradually over 14 days from ~5000% to ~500% and from 2000% to ~200% for 80Chit/20Col and 50Chit/50Col, respectively. This significant decrease in absorption capacity may be a consequence of chitosan migration

461 from scaffold to medium. Col-enriched compositions showed an almost time-
462 independent swelling profile which reached the maximum value within the first 24
463 hours, and then remained stable for up to 14 days, this being a sign of the material
464 stability of this composition in physiological medium up to prolonged incubation time.
465 TPP treatment, alone or with EDAC, markedly decreased the swelling capacity of all
466 scaffolds at early incubation stage (up to 24 hours) and for the samples with the
467 maximum chitosan content over all the period (up to 14 days). This 80%-Chit
468 composition showed, however, time-independent material swelling profiles after EDAC
469 crosslinking, while 50%-Chit EDAC treated samples began to lose part of their PBS
470 absorption ability after 3 days of incubation. For both 50 and 80%Chit-based scaffolds
471 material swelling was increasing with the time of incubation when these matrices were
472 exposed to TPP or TPP+EDAC. In the case of 20Chit/80Col scaffolds, all crosslinked
473 samples showed a similar behaviour. Although a slight decrease in PBS absorption was
474 detected during the first hours for samples exposed to TPP-based treatments, similar
475 swelling levels were then reached for all XL methods after 14 days in PBS.
476
477 *3.4 Mechanical testing*
478 Fig.4 (A-C) shows stress-strain curves of scaffolds with different compositions
479 and crosslinking statuses. Young´s moduli calculated from the initial parts of these
480 curves are presented in Table 2.
481 The influence of composition on the mechanical properties of scaffolds was
482 analysed by comparing the values of Young´s modulus of Non-XL samples. Results in
483 Table 2 showed that the increase in chitosan from 20 to 50% in scaffold composition
484 resulted in significant enhancement (~50%) of the values of elastic modulus (from 4.5

to 6.9 kPa, for 20Chit/80Col and 50Chit/50Col, respectively) but the further increase in Chit content caused decrease (~20%) of this parameter.

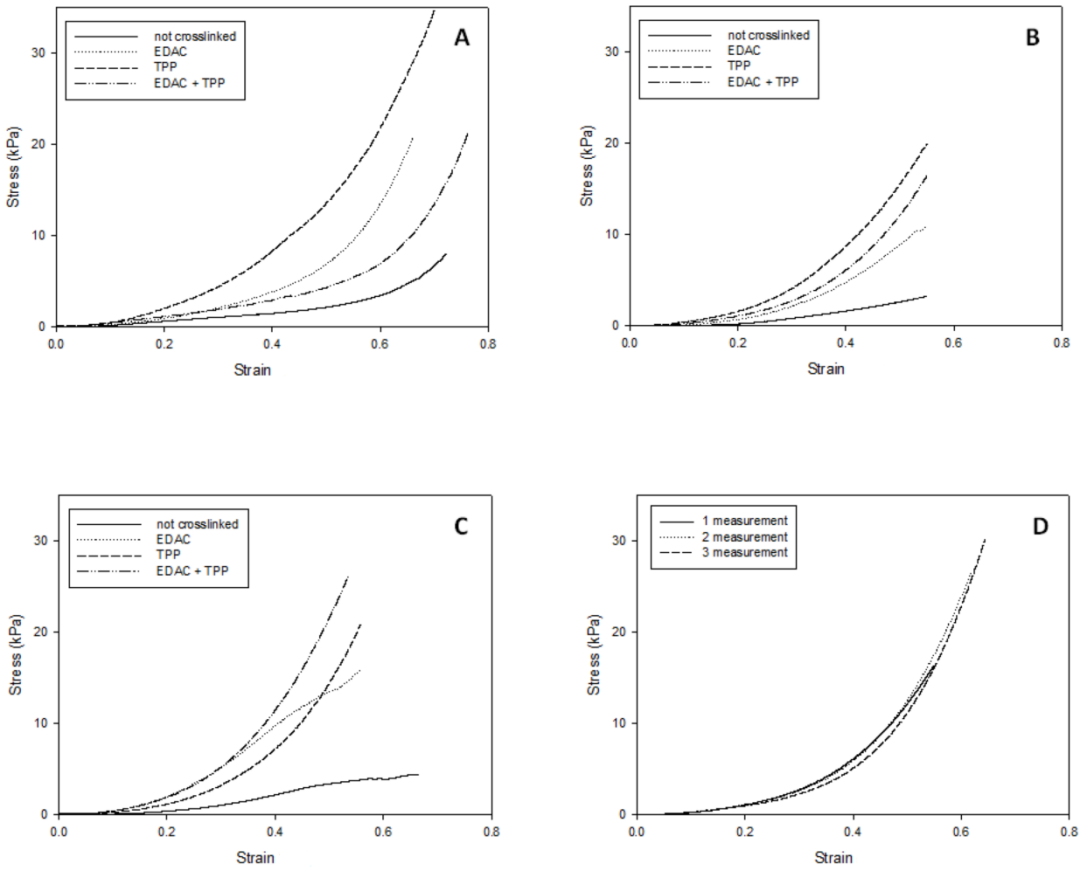


Fig. 4. Stress-strain curves for hydrated non-chemically crosslinked and crosslinked scaffolds tested under compression at 5 mm min^{-1} at room temperature: (A) 80Chit/20Col; (B) 50Chit/50Col and (C) 20Chit/80Col. (D) Typical profile of a stress-strain curve of three repeat tests of the same scaffold specimen (n = 5).

Crosslinking significantly enhanced the resistance to compression of all compositions but the degree of this rise was dependent on the component ratio and on the method employed (Table 2).

Table 2. Mechanical properties of scaffolds

Young´s modulus (kPa) (measured between 0.05-0.2 strain)			
	80Chit/20Col	50Chit/50Col	20Chit/80Col
Not crosslinked	4.5 ± 0.3	6.9 ± 0.8	5.5 ± 0.2

EDAC	9.9 ± 0.4*	12 ± 1*	23 ± 2*
TPP	20 ± 2*	11.7 ± 0.7*	15 ± 2*
EDAC+TPP	7.7 ± 0.5*	11 ± 1*	24 ± 3*

* Indicates a statistical difference in comparison to a non crosslinked sample of the same composition.

For the composition with the maximum chitosan content TPP provided the maximum increase (~4.5 fold) in Young's modulus. EDAC treatment was noticeably less effective for this composition as the compressive modulus increased only ~2.2 times after EDAC exposure and even less (~1.8 times) after combined treatment with EDAC+TPP . In the case of the collagen-enriched composition, an opposite influence of XL methods on scaffold stiffness was observed: EDAC and EDAC+TPP induced the highest improvement in mechanical properties (~4.5fold rise) while TPP exposure resulted in a more modest increase in the compression modulus (~2.7 times). For the 50Chit/50Col composition, all the treatments resulted in a similar increase in strength (~1.7fold).

In order to assess scaffold behaviour under successive compressions, the same sample specimens were repeatedly tested at least three times. These tests were carried out with all the crosslinked and Non-XL samples, excepting the 20Chit/80Col± EDAC-XL samples, and the 50Chit/50Col EDAC-XL composition. In the case of these latter systems, the structure cracked after the first compression, which indicated that these compositions produce the most fragile matrices. Typical stress–strain curves corresponding to the other samples (Fig.4D) showed no significant changes in their profiles after successive compressions (curves almost overlay each other in all stain intervals). This indicates good ability for structural recovery for these compositions and XL status.

3.5 In vitro collagenase and lysozyme degradation tests

Enzymatic degradation of chitosan/collagen scaffolds was studied by monitoring the residual mass percent of the samples after several days of incubation, separately, with the two enzymes collagenase and lysozyme. Figure 5 shows the percentage weight loss of chitosan/collagen sponges in collagenase and lysozyme solutions.

Non-XL scaffolds showed very poor resistance to enzymatic degradation in the presence of both enzymes. All samples lost almost 50% of weight within one hour in collagenase, while after 24 hours untreated samples were almost completely dissolved (80-100% mass loss).

In lysozyme, all compositions were more stable at the first hour of incubation, 50Chit/50Col sample being more resistant to digestion after prolonged incubation time (~60% mass loss after 10 days compared to 80-90% for the other two compositions). Crosslinking with TPP alone or in combination with EDAC significantly increased the resistance of all scaffold compositions to enzymatic degradation in the presence of lysozyme, while EDAC alone was effective only for 50% and 80% collagen-based scaffolds.

In collagenase, none of the employed treatments provided effective stabilisation of scaffolds with 80%-collagen content. For this composition the improvement in degradation stability was noticeable only for the very early stage of incubation in collagenase but after 3 days the scaffolds lost almost the same amount of material as Non-XL samples. Crosslinking, however, could, to some extent, protect the chitosan-enriched compositions against collagenase digestion, especially when TPP or TPP with EDAC were employed for scaffold treatment. The lowest values of mass loss were obtained for 50-50% composition after combined EDAC+TPP treatment (less than 40% degradation after 10 days of incubation).

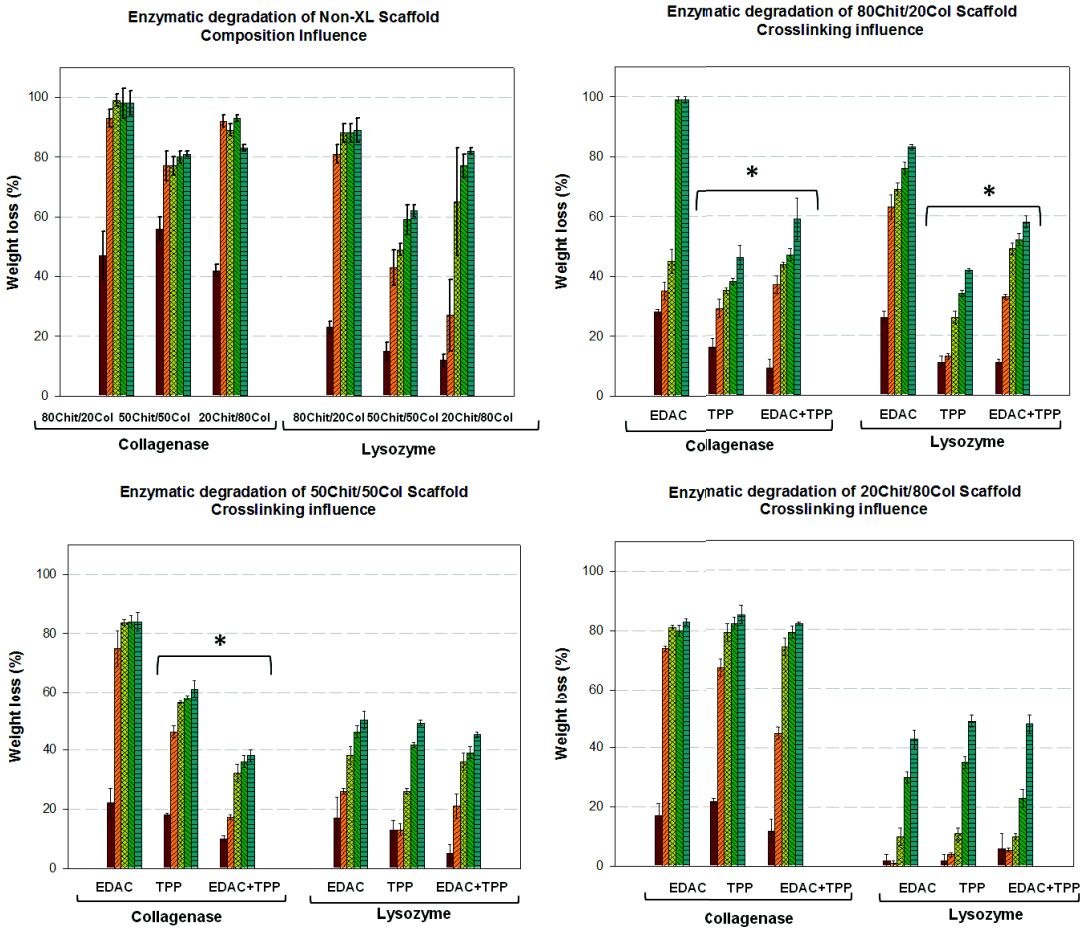
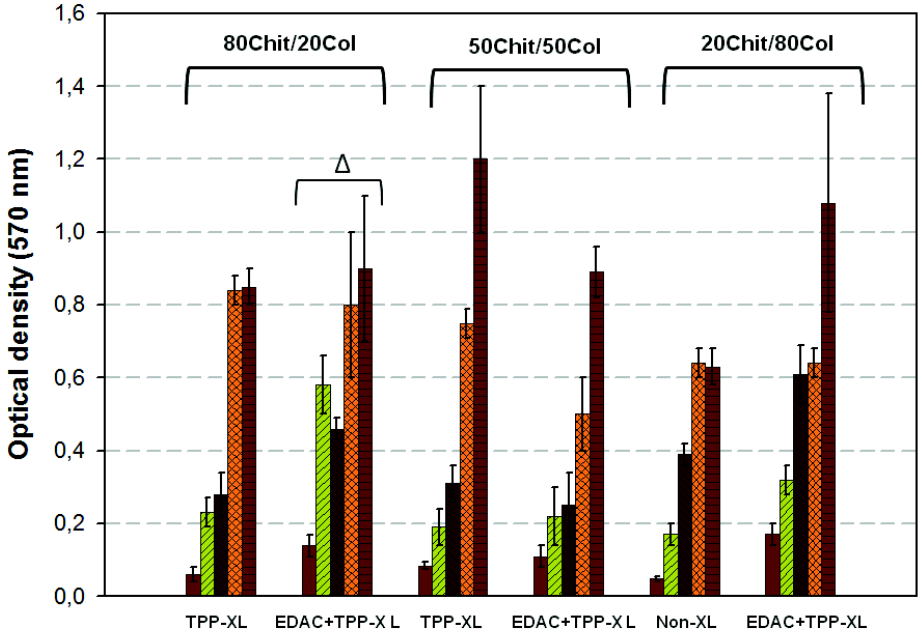


Fig. 5. Weight loss (%) in the presence of collagenase and lysozyme in PBS at 37ºC for scaffolds with different composition and crosslinking treatment: after 1 hour ( ), after 1 day ( ), after 3 days ( ), after 7 days ( ), after 10 days ( ). * means that results obtained from TPP and EDAC+TPP treated samples were significantly different ($p < 0.05$) from EDAC treated samples. (n = 4; bar charts represent standard deviation values).

3.6 Cell culture assays

The viability and proliferation of MCF-7 cells on selected chitosan/collagen scaffolds were evaluated over 5 days of incubation. Results were presented as the optical density, determined by MTT assay (Fig. 6). Control signals were subtracted from each composition's data.



561

562 Fig. 6. Proliferation trends and cell viability of MCF-7 cells on chitosan/collagen
563 scaffolds by MTT measurements: after 1h ( ), 24h ( ), 48h ( ), 96h (
564) and 120h ( ). Δ marks the composition without time-dependent growth. (n =
565 3; bar charts represent standard deviation values).
566
567

568 In general all scaffolds were found to be cytocompatible and adequate for cell
569 culturing. Cells started to proliferate on the scaffolds after 24 h of incubation in almost
570 all compositions, and continued growing for up to 4 days of incubation. Only the
571 80Chit/20Col EDAC+TPP-XL composition showed an irregular proliferation trend
572 without significant differences in the growth of cells from the first 24h of incubation.
573 On the contrary, cells continued growing until the end of the experiment on
574 50Chit/50Col and 20Chit/80Col EDAC+TPP-XL scaffolds.

575 In order to quantify the MCF-7 cell proliferation, specific growth rates (μ) were
576 determined by Eq. 9 and presented in Table 3. Doubling time values were also given in
577 this table. These parameters are usually determined to measure cell proliferation. It was
578 observed that the highest cell proliferation rate and, consequently, the lowest doubling

time were achieved in the case of 50Chit/50Col TPP-XL samples (Table 3). This composition seemed to be the most suitable for obtaining fast and time-dependent cell growth. However, this composition was followed closely by 80Chit/20Col TPP-XL, 50Chit/50Col EDAC+TPP and 20Chit/80Col Non-Crosslinked scaffolds which also produced good results in the parameters of cell proliferation.

Table 3. MCF-7 cell growth kinetic parameters.

Composition	Specific growth rate (μ, h^{-1})	Doubling time (td, h)
80Chit/20Col TPP	0.0155 (0.940)	44.7
80Chit/20Col EDAC+TPP	0.0046 (0.999)	150.7
50Chit/50Col TPP	0.0190 (0.999)	36.5
50Chit/50Col EDAC+TPP	0.0145 (0.960)	47.8
20Chit/80Col Non-Crosslinked	0.0147 (0.937)	47.1
20Chit/80Col EDAC+TPP	0.0120 (0.960)	57.8

Value in parenthesis means r^2 *(r^2 : correlation coefficient resulted after linear fitting of values obtained in the graphical representation of time (X) and cell viability (absorbance ,Y). Exponential growth was assumed)*

4. DISCUSSION

The main objective of this study was to determine the effect of changing composition and crosslinking methods on both the essential material properties and the biological suitability of scaffolds obtained from different collagen-chitosan mixtures. Three compositions, covering a wide range of ratios were selected for scaffold production, namely, Chit/Col = 80/20, 50/50 and 20/80 (wt%/wt%). As previously explained, these compositions were chosen with the aim of inducing different kinds of interactions between two polymer components according to their fractions in the blend. The extent of complexation reactions and the influence of different crosslinking treatments were evaluated in terms of structural properties, swelling/dissolution kinetics, mechanical properties and also biocompatibility of the resultant scaffolds.

4.1 Morphology

603 Chitosan-collagen blends were processed, using lyophilisation, to produce
604 homogeneous, highly porous (porosity ~96-99%) isotropic scaffold architectures with
605 pore diameters in the range 120-300μm. The values of pore size were slightly dependent
606 upon the polymer ratios which increased with increase of Col content. Crosslinking
607 does not affect significantly scaffold morphology with the exception of those matrices
608 obtained with the highest chitosan content and then treated with TPP, where a
609 significant ($p<0.05$) reduction in the pore size was observed. Since TPP acts through
610 ionic interactions with protonated amino groups of chitosan (Denkbas & Ottenbrite,
611 2006), this reduction in pore size could be the result of inter-molecular bond formation,
612 which is more pronounced in chitosan-enriched samples (80Chit/20Col).
613 Porosity in some cases was also affected to some extent by the crosslinking.
614 Porosity index is dependent upon the relative density which, in turn, is a function of the
615 volume of the sponge and the mass of its struts. In general, volume shrinkage may be
616 expected if a crosslinking agent alters the relative position of scaffold struts by pulling
617 them closer to each other, and so producing structural densification. Formation of inter-
618 fibre bonds during TPP treatment may explain volume shrinkage especially for the TPP-
619 XL Chit-enriched composition (80Chit/20Col).
620 Variation in mass is also a phenomenon which is frequently observed after
621 different crosslinking procedures (Grover, Cameron & Best, 2012): strut mass may
622 decrease in the case of material dissolution during the crosslinking process, or increase
623 if the crosslinking reagent forms part of the linkage and is in this way completely or
624 partially incorporated into the scaffold composition. Carbodiimide chemistry works by
625 promoting bonds between carboxylic and amine groups of the adjacent polypeptide
626 chains without entering into the scaffold structure, therefore mass increase is not
627 anticipated after this treatment (Olde Damink, Dijkstra, van Luyn, van Wachem,

628 Nieuwenhuis & Feijen, 1996b). TPP, however, uses its negatively charged phosphate
629 ions to establish ionic interactions with positively charged amino groups of chitosan.
630 This leads to the incorporation of the TPP molecules into the scaffold composition
631 which should affect (increase) scaffold mass. At the same time, during crosslinking by
632 any method, some mass loss may be expected as a result of polymer dissolution and
633 migration of unattached polymer chains into the crosslinking/washing media
634 (water/ethanol mixtures). Competition between these two processes, influencing
635 scaffold mass in an opposing way, may explain the results of scaffold weight change
636 after cross-linking: increase for Chit-enriched scaffolds mainly after TPP-base
637 treatments and decrease in the case of 80% Col-containing compositions (Table 1).

638 Changes in volume and mass should, in turn, affect the relative density values
639 and, consequently, scaffold porosity after XL procedures. Despite the observed
640 significant differences ($p<0.05$) in scaffold mass and volume (Table 1) after
641 crosslinking, especially those based on the TPP technique, a real effect on matrix
642 porosity was confirmed only in the case of the chitosan enriched (80Chit/20Col) TPP-
643 XL composition. These changes were also observed in the SEM images of this sample
644 (Fig.1). For the remaining scaffolds, the detected variations were not significant, which
645 could be result of combination and compensation of two opposite effects: volume
646 shrinkage and mass changes after the crosslinking treatment.

647 In spite of the observed dimensional alterations, these treatments do not
648 produced any noticeable detrimental effect on scaffold morphology: the pore size was
649 within a range suitable for the growth of the variety of cell lines (myocytes, endothelial
650 cells, fibroblasts, etc.), as reported in the literature (Gerdes, Moore, Hines, Kirkland &
651 Bishop, 1986; Radisic & Vunjak-Novakovic, 2005; Wang et al., 2010a) and the porosity
652 level, exceeded 90%, fell into the interval recommended for correct *in vitro* cell

adhesion, growth and reorganization, and also as the necessary space for *in vivo* neovascularisation (LeGeros, LeGeros & 1995). This means that the morphology of all matrices was appropriate for their potential use in different TE applications.

4.2 Dissolution behaviour

The resistance of scaffolds to dissolution, as expected, was strongly dependent upon composition and crosslinking treatment. In the case of non-XL samples, the better resistance to dissolution of collagen enriched scaffolds may be explained by a self-cross-linking phenomenon, due to PA/PC complex formation, which should be favoured in composite scaffolds with appropriate, near to stoichiometric ratios (Sionkowska, Wisniewski, Skopinska, Kennedy & Wess, 2004). 20Chit/80Col composition shows an almost stoichiometric weight percentage between its components, according to reports by Taravel and Domard (Taravel & Domard, 1996), followed next by the 50Chit/50Col sample. The stronger component interactions, as expected in these matrices, could explain the observed stability of 20 and 50%-Chit-based scaffolds (Fig.2) towards dissolution. At prolonged incubation times (14 days), however, these PA/PC complexes might be not strong enough to resist hydrolysis, which may explain the increase in dissolution of these compositions over time. Differences in mass loss between 80%Chit samples and those of lower chitosan content (50 and 20%) decreased from ~40%, after the third day in PBS, to 23% and 13% for 50 and 20% Chit containing matrices, respectively, at the end of testing (14 days).

Chemical crosslinking manages to stabilise almost all scaffold compositions in biological media but to a different degree. The most significant effect of all crosslinking methods on scaffold stabilisation was observed for Chit-enriched composition (80Chit/20Col), the TPP treatment being, as expected, the most effective, especially in combination with EDAC. This may be explained by the fact that the

679 component ratio in 80Chit/20Col samples is too far from stoichiometric, which does not
680 benefit PA/PC complex formation. This component proportion, however, may promote
681 a hydrogen-bonding type complex (HC) which is favoured in matrices with a great
682 excess of chitosan, as reported (Taravel & Domard, 1993, 1995, 1996). It was shown in
683 the PA/PC complex that the triple helix Col structure is preserved, and even reinforced,
684 by the presence of chitosan but, on the other hand, in the HC complex chitosan chains
685 seem to induce destabilisation of the triple helix organization and to denature the
686 collagen (Taravel & Domard, 1995). This means that untreated 80%Chit based
687 composition should be the least stable compared with those with less Chit content, and
688 as a result might be more favoured from the "external" linkage. The fact that chitosan,
689 the major component (80%) of this composition, interacts mainly with TPP explains the
690 strongest input of this treatment to the enhancement of matrix strength. It seems that
691 EDAC additionally contributes to scaffold stability by promoting links in polypeptide
692 chains of the remaining (20%) collagen component. For 50 and 20% Chit-based
693 scaffolds the crosslinking effect on dissolution was quite small and became noticeable
694 only from the third incubation day onwards when the differences between mass loss of
695 Non-XL and XL samples become more significant, especially for 50Chit/50Col
696 samples. In the case of 20Chit/80Col scaffolds, no significant differences were observed
697 between Non-XL and TPP-XL samples while EDAC+TPP could slightly contribute to
698 scaffold resistance to dissolution. These results may be explained by a clear self-
699 crosslinking effect, due to PA/PC complex formation, in both 50 and 20% Chit-based
700 scaffolds. So, while crosslinking can to some extent improve resistance to dissolution of
701 these self-cross-linked compositions, especially at prolonged time in an aqueous
702 environment, the influence of chemical treatments was little, if any, in the case of
703 80Col%, particularly over short incubation times.

4.3 Swelling properties

The ability of scaffolds to hold biological fluids is a very important factor in the evaluation of scaffold suitability as cell delivery vehicles for different in *vivo* and *in vitro* TE procedures (Engler, Bacakova, Newman, Hategan, Griffin & Discher, 2004; Park, Lee, Lee & Suh, 2003). Swelling characteristics indicate how cell culture medium may be absorbed during culture and how the scaffold may behave *in vivo*. The results of PBS uptake studies showed that overall swelling characteristics of scaffolds and the adsorption properties of struts alone were highly dependent on scaffold composition and crosslinking treatment. It was revealed that Chit-enriched (80%Chit) Non XL samples admitted the highest amount of media into their structures at an early incubation stage, most probably due to a lower level of interaction with their polymeric network (less PA/PC complex than in other compositions, as already described). The steepest descent in overall swelling after 24 hours incubation, and the lowest values of media uptake at the end of the experiment of this 80% Chit composition, may be a consequence of pore collapse due to lack of structural strength of its 3D matrix. Material absorption properties of this composition showed the same tendency as overall fluid uptake: the highest values of strut swelling at an early stage of incubation, most likely attributable to the higher hydrophilicity of chitosan (80% in the mixture) in comparison to collagen, and an abrupt decrease in material swelling over time as a result of strut dissolution and diffusion of weakly-bonded polymeric chains into the media.

Higher level of inter-molecular self-crosslinking in Col-enriched compositions in comparison with 20% Col samples, resulting in more tight and entangled internal structures, caused lower values of overall and material swelling at short incubation time to 50 and 80% Col Non-XL compositions. The increase of overall swelling profiles of

20Chit/80Col scaffolds with time may be explained by the highest extent of self-complexing in these matrices which provides them with stable media-holding characteristics over a prolonged incubation period. Gradual increase in absorption capacity during incubation may be a consequence of strut swelling, leading to loosening of the polymeric network which should increase its overall swelling capacity.

The influence of the crosslinking method on PBS uptake properties of composite scaffolds may also be explained by the extent of complex formation reactions in the matrices with different component ratios. Swelling behaviour of 80% and 50% chitosan-based scaffolds was more affected by chemical treatment, especially at prolonged incubation times. The descending profile of overall swelling after 24hours incubation of Chit-enriched EDAC-XL samples and the ascending tendency of these matrices after TPP+EDAC treatments, showed the importance of Chit-TPP interactions in the enhancement of the structural strength of their less self-bonded networks. Strut swelling at prolonged incubation time, leading to relaxation of chemically treated structures, may explain the rise in the degree of swelling with increase in time. For 20Chit/80Col scaffolds the highest value of fluid uptake at 14 days incubation (an almost twofold increase compared to Non-XL sample and to any other XL composition) pointed to a higher level of stability of bonds formed by crosslinking compared with those achieved by complexing reactions between chitosan and collagen. Crosslinking treatments decreased the level of the swelling capacities of the material itself at the early stages of incubation (up to 24 hour) for all compositions. This may be explained by a decrease in hydrophilicity of scaffold material after crosslinking due to (a) the consumption of groups involved in water uptake during the crosslinking process (carboxylic and amino groups) and (b) restraint in the ability to swell by formation of additional bonds. These results are in agreement with reports (Charulatha & Rajaram,

754 2003; Rehakova, Bakos, Vizarova, Soldan & Jurickova, 1996) showing that with an
755 increase in crosslinking densities swelling of material itself of Col-based scaffolds
756 decreased.
757
758 *4.4 Mechanics*
759 The development of scaffolds with adequate mechanical properties is one of the
760 greatest challenges in the design of tissue engineering constructs. Intense research has
761 shown that scaffold compressive properties, such as Young's modulus and the
762 compressive strength, have a great impact on the cellular activity during culturing.
763 Appropriate compressive properties prevent scaffolds from collapsing as a result of
764 cellular action within their matrices (Harley, Leung, Silva & Gibson, 2007; Horan et al.,
765 2005). On the other hand, it is clear that scaffold mechanics should be adjusted to
766 closely resemble the environment of extracellular matrices in order to encourage cells to
767 generate new artificial tissue and enhance repair. This demonstrates the importance of
768 developing strategies to modulate scaffold mechanics according to the biological
769 requirements of a specific application. In this work the variety of compressive
770 properties of 3D matrices were achieved by changing component ratios in Chit/Col
771 scaffolds and by variations in their crosslinking procedures. Compression tests
772 conducted in this study on the effect of composition on Young's modulus revealed that
773 the introduction of chitosan to collagen significantly increases the values of this
774 parameter for all composite scaffolds compared with pure Non-XL Col samples
775 (1.2kPa) which were produced and tested for comparison purposes. After EDAC
776 treatment of Chit/Col scaffolds under optimum crosslinking conditions (EDAC
777 11.5mg/ml, molar ratios EDAC/NHS/COO^-(Col) = 5/2/1)(Olde Damink, Dijkstra, van
778 Luyn, van Wachem, Nieuwenhuis & Feijen, 1996a; Park, Park, Kim, Song & Suh,

779 2002) their Young's modulus increased to 6.2kPa (data not shown) and reached the
780 same level as for Non-XL Col-Chit composite matrices (between 4.5 and 6.9kPa). This
781 revealed the importance of electrostatic interactions between scaffold components in
782 blended compositions in order to enhance matrix compressive properties. The maximum
783 value of elastic modulus was achieved when polymers were combined in equal
784 proportions (1:1 Chit/Col w/w%). This indicates that 50Chit/50Col composition
785 provides maximum stiffness to scaffold struts probably by the higher degree of
786 formation of a complex between Col and Chit. The increase of compressive modulus of
787 collagen-chitosan composite scaffolds was reported in the literature where better
788 material stabilisation in terms of compression was achieved at a ratio of 1:2 chitosan-
789 collagen (Arpornmaeklong, Pripatnanont & Suwatwirote, 2008; Chicatun et al., 2011).
790 Crosslinking usually increases the stiffness of the biopolymer matrices by
791 introducing additional linkage within the polymeric network which, in turn, enhances its
792 resistance to dimensional changes under compression (Olde Damink, Dijkstra, van
793 Luyn, van Wachem, Nieuwenhuis & Feijen, 1996a; Park, Park, Kim, Song & Suh,
794 2002). A similar effect of chemical treatment on scaffold compression properties was
795 observed in this study. The rise in modulus value was highly dependent upon the
796 composition and crosslinking procedure. The results may be logically explained by
797 differences in crosslinking degrees achieved in each composite sample exposed to TPP
798 or EDAC-based treatments. As has already described, a scaffold with excess Chit in its
799 composition should be more efficiently crosslinked by the TPP procedure, while
800 matrices with higher Col content should be more favoured by EDAC-base treatment.
801 The results of mechanical testing corroborate this explanation, as a higher level of
802 improvement in Young's modulus (~4.5 fold increase) was observed for Chit-enriched
803 composition (80%Chit) after TPP treatment. EDAC, alone or with TPP, was less

804 effective and provided a lower (less than 2fold) rise in this parameter. In contrast, for
805 80% Col scaffolds the most significant increase in compressive modulus (~4.4 fold) was
806 observed after EDAC-based treatments. It is worth mentioning that although
807 crosslinking did not produce any significant changes in term of dissolution resistance
808 and swelling behaviour for the 20Chit/80Col composition, mechanical properties were
809 highly dependent upon the crosslinking procedure. So it may be concluded that in
810 general, scaffold stiffness could be effectively modulated by both composition and
811 crosslinking in order to tailor the mechanics of these matrices towards specific TE
812 application.
813
814 *4.5 Enzymatic degradation*
815 It is known that bacterial collagenase (*Closrium hisrolyticum*) specifically
816 cleaves the peptide bond on the amino side of glycine in collagen sequences (Lee, Park,
817 Hwang, Kim, Kim & Sub, 2001; Weadock, Miller, Keuffel & Dunn, 1996) while
818 lysozyme hydrolyses and metabolises chitosan molecules, leading to the release of
819 amino-sugars (Seda Tigli, Karakecili & Gumusderelioglu, 2007). Therefore it may be
820 anticipated that Non-XL Chit enriched compositions should be degraded more in
821 lysozyme containing media while scaffolds with higher collagen content should be more
822 susceptible to the presence of collagenase (Harper & Kang, 1970). On the other hand, it
823 may be expected that polyelectrolyte complex formation, being more favoured in a Col-
824 enriched composition, could to some extent protect composite scaffolds from enzymatic
825 digestion. Results showed, however, very poor stability of all untreated compositions in
826 the presence of enzymes, especially collagenase. Even 80%-Col samples, where almost
827 all polymeric content should be involved in polyelectrolyte complexation, were almost
828 completely dissolved after 24 hours in the presence of collagenase, in a way similar to

829 other compositions. This may indicate that linkage resulting from Chit/Col ionic
830 interactions was not strong enough or/and the location of these bonds was not adequate
831 to protect collagen sequences from enzymatic attack.

832 Crosslinking usually improves the biostability of scaffolds in the presence of
833 enzymes. EDAC treatment is reported as being highly efficient in increasing the
834 resistance of collagen-based scaffold to collagenase digestion (Ma et al., 2003; Powell
835 & Boyce, 2007; Zhu, Liu, Song, Jiang, Ma & Cui, 2009) Our results revealed, however,
836 rather a limited improvement in scaffold stability in collagenase containing media after
837 EDAC-based treatment, especially for 80% Col-containing compositions. Moreover, it
838 was observed that none of the crosslinking methods could stabilise these Col-enriched
839 scaffolds in the presence of collagenase. This may indicate that polyelectrolyte complex
840 formed in Col-Chit scaffold with stoichiometric component ratio is responsible for low
841 efficiency of EDAC and TPP treatments towards collagenase digestion. The amount of
842 free amine/carboxylic groups on collagen left after PA/PC complex formation may be
843 insufficient to achieve the formation of a high density network after treatment. The fact
844 that pure Col scaffolds treated with EDAC showed very high resistance to collagenase
845 digestion, by losing only ~8% weight after 14 days incubation (results not shown),
846 contributes to this supposition. Chemical treatments, however, could stabilise 80% Col
847 composition in the presence of lysozyme, which indicates that (a) some additional
848 modification of chitosan takes place after crosslinking procedures and (b) crosslinks
849 formed could prevent scaffolds from hydrolysis by lysozyme. In the case of the other
850 two compositions, where Chit/Col ratios were further from stoichiometric, chemical
851 crosslinking could noticeably contribute to scaffold stability in the presence of both
852 collagenase and lysozyme. It seems that chitosan (polymer in excess) managed to form
853 additional bonds, mainly with TPP, which may explain the better efficiency of TPP–

854 based methods in scaffold stabilisation in the presence of both collagenase and
855 lysozyme. The network formed by crosslinking, in addition, should prevent the
856 enzymes, by steric hindrance, from accessing the active sites on the collagen and
857 chitosan molecules, or by holding enzyme-cleaved chain sections intact.
858
859 *4.6 Cell studies*
860 Collagen and chitosan have been widely used as materials for the preparation of
861 scaffolds where cells could be seeded and proliferate successfully, either used separately
862 (Chen et al., 2012; Dhiman, Ray & Panda, 2004) or in combination (Zhu, Liu, Song,
863 Jiang, Ma & Cui, 2009). In spite of the expected good cytocompatibility of scaffolds
864 based on Chit/Col combination, the evaluation of cell viability is necessary to assess
865 scaffold´s potential for TE applications. Therefore, MTT assay was performed to pre-
866 evaluate the proliferation rate of cells since this method has been extensively used for
867 this purpose and reveals the extent of cellular metabolism and viability of the cells
868 (Chung & Chang, 2010; Jin, Chen, Karageorgiou, Altman & Kaplan, 2004).
869 Additionally, MCF-7 line has been also commonly selected to test the compatibility of
870 different types of natural polymers with biomedical applications (Chen et al., 2012;
871 Wang et al., 2010b).
872 Taking into account the results obtained from *in vitro* studies, it was
873 hypothesized that TPP-XL and EDAC+TPP-XL samples for 80Chit/20Col and
874 50Chit/50Col compositions, and Non-XL and EDAC+TPP-XL for 20Chit/80Col
875 mixture, would offered promising and suitable bulk characteristics for cell culture. The
876 selection of Non-XL 20Chit/80Col scaffolds was based on the advantage which would
877 be offered by the self-crosslinking phenomenon observed in this composition for cell
878 culture studies. This particular behaviour would avoid the need for using additional

879 chemical reactants to obtain a stable 3D scaffold, which could be considered as an
880 advantage to obtain more biocompatible structures.

881 Our results confirmed the foreseen good compatibility of all scaffolds seeded
882 with MCF-7 cells. The MTT reduction, which is considered as a marker reflecting
883 viable cell metabolism, increased up to 4-5 days without significant decrease in any
884 composition. Therefore, prolonged exposure of cells to Chit/Col scaffold did not result
885 in cell death. Our results are in accordance with other works, where a similar tendency
886 of MCF-7 viability was obtained after their inoculation in pure chitosan matrices for 5
887 days of culture period (Dhiman, Ray & Panda, 2004, 2005) and in pure collagen
888 scaffolds up to 13 days (Chen et al., 2012). Additionally, the good cytocompatibility of
889 Chit/Col composites was also ensured by Fernandes and co-workers using MC3T3
890 osteoblasts ((Fernandes, Resende, Tavares, Soares, CastroI & GranjeiroI, 2011) and by
891 Tsai and collaborators with WS1 skin fibroblasts (Tsai et al., 2007).

892 Our results were especially remarkable in the case of the 50Chit/50Col-TPP
893 composition. Cells grew throughout these scaffolds with the fastest growth rate and
894 doubled the cell population in the lowest time. Similar growth rates and doubling time
895 values were obtained by Seda and co-workers, who seeded fibroblasts on pure chitosan
896 scaffolds for 5 days (Seda Tigli, Karakecili & Gumusderelioglu, 2007). However, they
897 detected a reduction in cell number after 3 days of culture period as well as some
898 difficulties in achieving adequate cell growth throughout the matrix with the smallest
899 pore size. In contrast, our scaffolds did not show any reduction in cell viability, even
900 using the composition with the smallest pore size (80Chit/20Col-TPP-XL, 120 ± 42
901 μm). Consequently, the good results obtained for the screening of cell viability might
902 indicate that the assayed chitosan/collagen scaffolds could be used for further *in vitro*

and *in vivo* studies to better evaluate which of the systems already prepared offer the best characteristics for use in TE applications.

5. CONCLUSION

In this study, various chitosan/collagen scaffolds have been developed and characterised in terms of some of the most important physicochemical properties to be used with TE applications. According to changes in composition and crosslinking methodology, a wide range of scaffolds with different stability, degradation and swelling properties were obtained. These scaffolds should offer great versatility for selecting the most suitable structure for specific applications in the future. However, the most significant results relate to the 20Chit/80Col composition, whose self-crosslinking phenomenon eliminates the need for using additional chemical reactants, so making the product much more biocompatible. Furthermore, the use of crosslinking reagents, especially TPP or EDAC+TPP, seems to be essential in stabilizing the scaffolds where the chitosan proportion increased. Generally, all scaffolds seeded with MCF-7 allow a gradual increase in the number of cells in 3D structures for up to 5 days. These results showed the possible application of the developed scaffolds in TE, offering the possibility of tailoring their properties according to their final biological use and the requirements of the tissue under consideration.

5. ACKNOWLEDGEMENTS

The authors are grateful to Dr. von Kobbe (Chimera Pharma of Bionostra Group) for the gift of MCF7 cells. The financial support of the Ministerio de Ciencia e Innovación of Spain (FIS PS09/01513), and the FPI grant from UCM to A. Martínez are gratefully acknowledged.

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929 **6. REFERENCES**

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